



# CD4 and MHC-I downregulation are conserved in primary HIV-1 Nef alleles from brain and lymphoid tissues, but Pak2 activation is highly variable

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## Abstract

HIV-1 compartmentalization in the CNS has been demonstrated for *gag*, *pol*, and *env* genes. However, little is known about tissue compartmentalization of *nef* genes and their functional characteristics in brain. We have cloned 97 *nef* genes and characterized 10 Nef proteins from autopsy brain and lymphoid tissues from 2 patients with AIDS and HIV-1-associated dementia. Distinct compartmentalization of brain versus lymphoid *nef* genes was demonstrated within each patient. CD4 and MHC-I downregulation were conserved in all tissue-derived Nefs. However, MHC-I downregulation by brain-derived Nefs was weaker than downregulation by lymphoid-derived Nefs. The motifs KEEE- or EKEE- at the PACS-1 binding site represented brain-specific signature patterns in these 2 patients and contributed to the reduced MHC-I downregulation activity of brain-derived Nefs from these patients. Pak2 association was highly variable in Nefs from both patients. Three of 10 tissue-derived Nefs coimmunoprecipitated activated Pak2, with strong association demonstrated for only 2 Nefs. The ability of Nef to associate with activated Pak2 did not correlate with brain or lymphoid tissue origin. *Nef* genes from viruses isolated from brain by coculture with PBMC were not closely related to sequences amplified directly from brain tissue, suggesting that viral selection or adaptation occurred during coculture. This study of tissue-derived HIV-1 Nefs demonstrates that CD4 and MHC-I downregulation are highly conserved Nef functions, while Pak2 association is variable in late stage AIDS patients.

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**Keywords:** Human Immunodeficiency Virus type 1 (HIV-1); Nef; Brain; Lymph node; CD4; MHC-I; Pak2

## Introduction

The Nef protein of human and simian immunodeficiency viruses (HIV-1, HIV-2 and SIV) is an important determinant of progression to AIDS. Nef is required for maintenance of high viral load and disease induction in SIV-infected rhesus monkeys (Du et al., 1995; Kestler et al., 1991). Furthermore, expression of the HIV-1 *nef* gene in transgenic mice produces AIDS-like disease (Hanna et al., 1998; Skowronski et al., 1993). Nef-defective HIV-1 has been associated with long-term non-progression in infected individuals (Dyer et al., 1999; Kirchhoff et al., 1995; Learmont et al., 1992). These findings suggest that Nef is important for viral replication and pathogenicity in vivo.

HIV-1 Nef is a 27 kDa membrane-associated cytoplasmic protein expressed during early stages of viral replication. Well-studied functions of Nef include downregulation of CD4 and MHC-I, enhancement of viral infectivity, and modulation of cellular signaling pathways (reviewed in (Arold and Baur, 2001; Bour and Strebel, 2000; Fackler and Baur, 2002; Geyer et al., 2001; Wei et al., 2003)). Nef downregulates CD4 by linking cell surface CD4 to the adaptor protein complex of clathrin-coated pits (Bresnahan et al., 1998; Greenberg et al., 1998a) and transferring CD4 to COP-I for transport to lysosomes (Piguet et al., 1999a). Downregulation of cell surface CD4 enhances HIV-1 replication and infectivity, virion production and release, and prevents superinfection of infected cells (reviewed in (Harris, 1999; Piguet et al., 1999b; Lama, 2003)). MHC-I downregulation is achieved by targeting of MHC-I to the endosome-to-Golgi sorting pathway governed by PACS-1 (phospho-furin acidic cluster sorting protein-1) (Doms and

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Trono, 2000). Nef motifs critical for MHC-I downregulation are the acidic EEEE<sub>62–65</sub> motif, which binds PACS-1 (Piguet et al., 2000), and the proline-rich SH3-binding domain (PxxP domain) (Blagoveshchenskaya et al., 2002; Greenberg et al., 1998b; Mangasarian et al., 1999). MHC-I downregulation may be important for protection of infected cells against lysis mediated by HIV-I-specific cytotoxic T lymphocytes in vivo (Collins et al., 1998; Swigut et al., 2004; Tomiyama et al., 2002; Yang et al., 2002). The mechanism by which Nef enhances viral infectivity is still controversial.

Nef expression enhances HIV-1 replication when resting peripheral blood mononuclear cells (PBMC) are infected and subsequently stimulated (Miller et al., 1995; Spina et al., 1994), most likely by activation of resting T cells through modulation of cellular signaling pathways (Alexander et al., 1997; Baur et al., 1994; Du et al., 1995; Schragar and Marsh, 1999; Simmons et al., 2001; Wang et al., 2000; Wu and Marsh, 2001). In vivo, the SIVmac239 *nef* gene enhances productive infection of resting memory CD4<sup>+</sup> T cells in the paracortical regions of lymph nodes, thereby increasing viral replication and pathogenicity (Sugimoto et al., 2003). The interaction between Nef and p21-activated kinase 2 (Pak2), a cellular serine/threonine kinase, has been proposed to play an important role in T cell activation and AIDS progression in vivo (Arora et al., 2000; Lu et al., 1996; Nunn and Marsh, 1996; Renkema et al., 1999; Renkema et al., 2001; Sawai et al., 1996; Wei et al., 2005; Wolf et al., 2001). Pak2 is involved in the regulation of several cellular processes including cytoskeleton rearrangement, cell morphology, motility, apoptosis and gene transcription and is activated in response to a variety of cellular stresses (reviewed in (Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999)). Nef–Pak2 association has also been demonstrated in primary macrophages (Brown et al., 2004). The PxxP domain of Nef, which is required for interaction with the SH3 domains of Src tyrosine kinases and other signaling molecules, is required for Pak2 activation (Khan et al., 1998; Manninen et al., 1998; Wiskerchen and Cheng-Mayer, 1996). A cluster of residues on the surface of the Nef core domain near F191 and H89 is also critical for association with activated Pak2 (Agopian et al., 2006; Foster et al., 2001; O'Neill et al., 2006), and may form part of a unique binding surface specifically involved in Nef–Pak2 association.

In addition to enhancing T cell activation, Nef also activates signaling pathways in monocyte-derived macrophages. Macrophages play an important role in HIV-1 transmission and AIDS pathogenesis, and are the primary cellular reservoir for HIV-1 infection in the CNS (Dunfee et al., 2006; Gonzalez-Scarano and Martin-Garcia, 2005; Verani et al., 2005). HIV-1-infected macrophages release inflammatory cytokines including MIP-1 $\alpha$ /CCL3, MIP-1 $\beta$ /CCL4, and other factors (e.g., IL-16 and soluble forms of CD23 and ICAM-1) that contribute to leukocyte recruitment and activation in a Nef-dependent manner (Olivetta et al., 2003; Swingler et al., 1999, 2003; Zhao et al., 2004). The PxxP domain and motifs involved in CD4 downregulation in Nef are important for efficient viral replication in macrophages (Brown et al., 2004). Nef-expressing macrophages transplanted into the brain of rats induce

monocyte/macrophage recruitment, TNF- $\alpha$  expression, astrogliosis, and cognitive defects (Mordelet et al., 2004). Furthermore, SCID/NOD mice injected with Nef-expressing viral vectors exhibited evidence of neuronal injury and neurological disease (van Marle et al., 2004). HIV-1-infected and/or activated macrophages release soluble neurotoxic factors that induce neuronal injury and neurocognitive impairment (Gonzalez-Scarano and Martin-Garcia, 2005; Kaul et al., 2005). Nef may regulate production of some of these neurotoxic factors by stimulating macrophage activation (Mahlknecht et al., 2000; Olivetta et al., 2005; Vilhardt et al., 2002).

HIV-1 enters the CNS in the early stages of infection by trafficking across the blood–brain barrier within infected monocytes and possibly lymphocytes (reviewed in (Williams and Hickey, 2002; Dunfee et al., 2006)). However, productive infection of macrophages and microglia is typically detected only after progression to AIDS. Genetic evolution of HIV-1 within the brain is distinct from that in lymphoid tissues and other organs (Chang et al., 1998; Donaldson et al., 1994; Gartner et al., 1997; Gorry et al., 2001; Hughes et al., 1997; Korber et al., 1994; Shapshak et al., 1999; van't Wout et al., 1998; Wong et al., 1997). Previous studies demonstrated CNS compartmentalization of the HIV-1 *gag*, *pol*, and *env* genes. Only one previous study analyzed the genetic compartmentalization of full-length *nef* genes in the CNS (van Marle et al., 2004). This study also found that 3 brain-derived *nef* genes differentially induced pro-inflammatory gene expression in astrocytes, indicating that Nef sequence variability could affect pathogenesis. However, little is known about functional differences of Nefs from brain versus Nefs from other tissues. Nefs in brain may be functionally divergent from those in blood or lymphoid tissues as a consequence of adaptation to different target cell populations and reduced immune surveillance. In particular, low CD4 expression on macrophages/microglia and reduced CTL surveillance in the brain could result in reduced selection pressure to maintain strong CD4 or MHC-I downregulation activity, respectively.

To investigate genetic and functional characteristics of Nefs in brain versus lymphoid tissues, full-length *nef* genes were amplified from the genomic DNA of autopsy brain and lymphoid tissues from 2 patients with late stage AIDS and HIV-1-associated dementia (HAD). Distinct compartmentalization of brain versus lymphoid *nef* sequences was demonstrated within each patient. CD4 and MHC-I downregulation activities were highly conserved among primary Nefs. However, MHC-I downregulation by brain-derived Nefs was weaker than downregulation by lymphoid-derived Nefs. The motifs EKEE<sub>65</sub> or KEEE<sub>65</sub> in the PACS-1 binding site were identified as brain-specific signature patterns in both patients, whereas most lymphoid Nefs had the motif EEEEE<sub>65</sub>. Mutagenesis demonstrated that Nefs with EKEE<sub>65</sub> or KEEE<sub>65</sub> had a reduced capacity to downregulate MHC-I. Nefs with EEEEE<sub>65</sub>. Higher frequencies of EKEE<sub>65</sub> or KEEE<sub>65</sub> motifs and reduced capacity to downregulate MHC-I in brain-derived Nefs from these 2 AIDS patients may reflect reduced immune selection pressures in the CNS compared to non-CNS tissues. Of 10 brain- and lymphoid tissue-derived Nefs, only 3 associated with activated

Pak2. The ability of Nef to associate with Pak2 did not correlate brain or lymphoid tissue origin. These findings indicate that CD4 and MHC-I downregulation are conserved in primary Nef alleles derived from brain and lymphoid tissues from late stage AIDS patients, while Pak2 activation is highly variable.

## Results

### *Amplification of nef alleles from brain and lymphoid tissues and brain- and lymph node-derived viral isolates*

To compare genetic and functional characteristics of Nef sequences from brain versus lymphoid tissues, we amplified *nef* genes from the genomic DNA of autopsy brain and lymph node tissues of 2 patients (MACS2 and MACS3) with late stage AIDS and HIV-1-associated dementia (Gorry et al., 2001). *Nef* genes were also amplified from the genomic DNA of spleen of patient MACS2. Phylogenetic analysis showed that sequences from both bulk and limiting dilution PCRs were well interleaved and did not show evidence of amplification bias (data not shown). To reduce potential effects of primer bias, 2 different primer sets were used in limiting dilution PCR reactions. Although some sequences were more commonly amplified by one or the other primer set, both primer sets amplified at least one *nef* sequence from every major branch of the phylogenetic trees (data not shown). A total of 51 *nef* sequences were amplified directly from MACS2 tissue (18 from brain, 22 from lymph node, and 11 from spleen) and 46 from MACS3 tissue (12 from brain, and 34 from lymph node). All *nef* sequences amplified directly from MACS2 and MACS3 tissues encoded full-length open reading frames.

In addition to amplifying *nef* genes directly from the tissue samples as discussed above, we also amplified *nef* genes from viral isolates obtained from coculture of PBMC with the same tissues. These isolates, MACS2-br, MACS2-LN, MACS3-br and MACS3-LN, were described previously (Gorry et al., 2001). For each viral isolate, we sequenced the bulk PCR product and 3 to 7 *nef* genes amplified from genomic DNA isolated from PBMC on day 7 post-infection with virus stock. Within each viral isolate, Nef clones were highly similar (98.3%–100% Nef predicted amino acid identity). All *nef* genes from viral isolate MACS2-br, including representative Nef clone 4G, contain an early frameshift that introduced a stop codon after only 61 amino acids. Clone MACS2-br-4G, could not be detected by Western blot and was non-functional in assays for CD4 and MHC-I downregulation and Pak2 activation (data not shown). Representative Nef clones matching the consensus sequence of each of the other viral isolates were chosen for further functional analysis. Sequences and functional analyses of representative viral isolate clones MACS2-LN-5C, MACS3-br-6I, and MACS3-LN-7D have been published (Agopian et al., 2006).

### *Nef sequences from viral isolates derived from brain-PBMC co-cultures are not representative of those in brain tissue*

To determine whether coculture of brain tissue with PBMC resulted in viral isolates representative of virus in the brain in vivo, we used phylogenetic analysis to compare the nucleotide

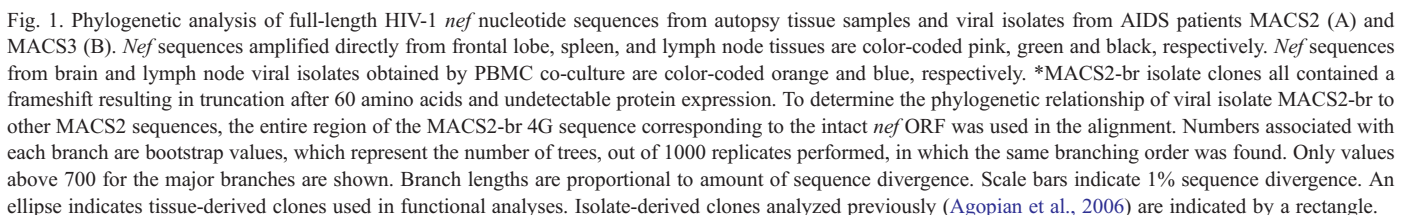
sequences of *nef* genes from PBMC/brain coculture-derived viral isolates with sequences of *nefs* amplified directly from the brain tissues. We found that *nef* sequences amplified from viral isolates derived by brain-PBMC coculture were not representative of *nef* sequences in brain tissue. Even after repair of the frameshift, the nucleotide sequence of *nef* clone 4G from viral isolate MACS2-br (shown in pink in Fig. 1A) is highly divergent from all other MACS2 sequences, including sequences amplified directly from the brain of MACS2. The sequence of *nef* clone 6I from viral isolate MACS3-Br is closely related to *nef* sequences from lymph node tissue and is highly divergent from *nef* sequences amplified directly from MACS3 brain. In contrast, the sequences of *nef* clones 5C and 7D from viral isolates MACS2-LN and MACS3-LN were closely related to *nef* sequences amplified directly from the DNA of MACS2 or MACS3 lymph node tissue. Together, these data indicate that *nef* sequences from primary viruses isolated by brain-PBMC coculture are not necessarily representative of *nef* sequences in the brain and suggest that viral selection or adaptation to PBMC may have occurred during brain coculture.

### *Nef sequences in brain versus lymphoid tissue are compartmentalized*

Phylogenetic analysis revealed distinct compartmentalization of tissue-derived brain versus lymphoid *nef* sequences within each patient (Fig. 1). *Nef* sequences amplified directly from 2 different sections of MACS2 frontal lobe tissue showed very little sequence diversity (Fig. 1A). The low amount of MACS2 brain *nef* sequence diversity is consistent with the low HIV-1 copy numbers in these MACS2 brain tissue samples. *Nef* sequences from MACS2 lymph node and spleen were more diverse and were well interleaved, indicating a lack of compartmentalization between these 2 lymphoid tissues. Thus, in patient MACS2, *nef* sequences are compartmentalized between brain and lymphoid tissues. In patient MACS3, 8 *nef* sequences amplified directly from brain were highly divergent from sequences amplified from lymph node tissue (Fig. 1B). These sequences comprise a branch of the MACS3 phylogenetic tree that is represented solely by *nef* sequences from brain tissue. Therefore we designated these 8 sequences as ‘true’ or ‘unique’ brain Nefs. Four *nef* sequences amplified from MACS3 brain tissue were identical or nearly identical to sequences amplified from lymph node. Thus, patient MACS3 demonstrates both compartmentalized brain *nef* sequences and *nef* sequences shared by lymph node and brain. Together, phylogenetic data from both late-stage AIDS patients MACS2 and MACS3 demonstrate compartmentalization of *nef* sequences in the brain versus lymphoid tissues.

### *Conservation of motifs in representative tissue-derived nef sequences*

To test for functional differences between Nef sequences from brain versus lymphoid tissue, 2 to 3 representative *nef* sequences from each tissue were selected (circled in Fig. 1). These Nefs were chosen so that all major branches of each





phylogenetic tree were represented. Fig. 2 shows an amino acid sequence alignment of these representative Nefs. Well-conserved motifs in these Nefs include the myristoylation signal (Geyer et al., 2001), the PxxP SH3-binding region (Saksela et al., 1995), the putative CD4 binding site (Grzesiek et al., 1996), and motifs in the flexible loop proposed to be important for association with the endocytic machinery including  $\beta$ -COP (Piguet et al., 1999a), adaptor proteins AP-1/2/3 (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998a) and vacuolar ATPase (Lu et al., 1998). More variable motifs in our primary Nefs are the PACS-1 binding motif (Piguet et al., 2000), residues 85, 89, 187, 188, and 191 important for Pak2 association (Agopian et al., 2006; O'Neill et al., 2006), and the FPD<sub>121</sub> motif implicated in thioesterase binding (Cohen et al., 2000). Analysis of Nef expression in 293 T cells by Western blotting suggests that except for 16N, these Nef proteins are expressed at levels similar to those of control Nef proteins SF2 and NL4-3 (see Figs. 3B, 4B, and 5A

and B). Nef 16N contains the variation T<sub>80</sub>I, which disrupts a consensus phosphate acceptor target for protein kinase C (Shugars et al., 1993). Mutation of this highly conserved threonine has been shown to reduce protein stability (Aiken et al., 1996). Although Western blotting with three different polyclonal sera suggests that 16N is expressed at lower levels compared to the other Nef alleles, we cannot rule out the possibility that 16N is well expressed but poorly recognized by these sera. All five MACS3 brain Nef sequences closely related to 16N carry this T<sub>80</sub>I variation.

#### CD4 downregulation is highly conserved

To examine the ability of brain and lymphoid Nef proteins to downregulate CD4, we co-transfected Nef and GFP plasmids into HIJ cells (MAGI cells expressing high levels of CD4) and analyzed cells for surface expression of CD4 by flow cytometry (Fig. 3). The LL<sub>164</sub>AA mutation, previously shown to abolish

	Myristoylation	CD4	PACS-1	PxxP
CONSENSUS_B	1 MGGKWSKRSVVGWPTVRERMRAEP----AADGVGAVSRDLEKHGAITSSNTAANNADCAWLEAQE-EEEVGFVPRPQVP			
MACS2FL_14A	1 .....S.....A.....R.....TL.....K-.....			
MACS2FL_14E	1 .....S.....A.....R.....TL.....K-.....			
MACS2LN_15A	1 .....GG..AA.....RAEP..E..PA.....R.....TL.....E.....			
MACS2LN_15F	1 .....GG..AS.....RAEP..E.....R.....L..VQ..E.....			
MACS2Sp_25E	1 .....GG..AS.....Q..RAEP..E..PA.....R.....TLT.....E.....			
MACS3FL_16N	1 .....C..G..SA.....ATEP..A.....P.T.....-K.....			
MACS3FL_16P	1 .....C..G..SA.....K.....ATEP..A.....Q.....P.T..N.....-			
MACS3LN_17A	1 .....CGLG..SA.....ATEP..A.....P.T.....E.....			
MACS3LN_17B	1 .....CGLG..SA.....ATEP..A.....P.T.....E.....			
MACS3LN_17F	1 .....CGLG..SA.....ATEP..A.....P.T.....Q.....			

	PxxP	85	89	CD4	thioesterase	$\beta$ -COP
CONSENSUS_B	76 LRPMTYKGAIDLSHFLKEKGGLEGLIYSQKRQDILDWVYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVEPEKVEE					
MACS2FL_14A	76 .....F.....R.....L...C.....EI..					
MACS2FL_14E	76 .....F.....L...C.....EI..					
MACS2LN_15A	81 .....F.....F.....W.....L...C.....V.....					
MACS2LN_15F	81 .....F.....Q.K.....N.....C.....V.....					
MACS2Sp_25E	81 .....F.....F.....W.....L.N.....L...C.....					
MACS3FL_16N	80 ....I..A...I.....R.....N.....V.....D.DQ..					
MACS3FL_16P	80 .....A...I.....R.....N.....S.....V.....D.D..					
MACS3LN_17A	81 .....A...M..L..D.....K..E.....N.....V.....D.DQ..					
MACS3LN_17B	81 .....A...M..L..D.....K..E.....N.....V.....D.DQ..					
MACS3LN_17F	81 .....A...M..L.....K..E.....N.....V.....D.DQ..					

	AP-1/2/3	V-ATPase	187-8	191
CONSENSUS_B	156 ANEGENNSLLHPMSLHGMDPPERVLVVKFDSRLAFHHMARELHPEYYKDC			
MACS2FL_14A	156 ..K....C.....E.....K....F.N.			
MACS2FL_14E	156 ..K....C.....E.....K....F.N.			
MACS2LN_15A	161 .....C.....Q.....K.....IH..H..I..K....F.N.			
MACS2LN_15F	161 .....C.....Q.....E..K.....Q..R..V..K....F.N.			
MACS2Sp_25E	161 .....C.....Q.....E..K.....Q..R..V..K....F.N.			
MACS3FL_16N	160 .T.....IC...E...G..M.....K...F...			
MACS3FL_16P	160 .T.....IC...E...G..M.....K...F...			
MACS3LN_17A	161 .T.....IC...E...K..M.....K...F...			
MACS3LN_17B	161 .T.....IC...E...G..M.....I..K....N.			
MACS3LN_17F	161 .T.....IC...E...G..M.....K...F...			

Fig. 2. Amino acid alignment of primary HIV-1 Nef clones with the Clade B consensus. The PACS-1 binding site, important for MHC downregulation (Piguet et al., 2000), and residues 85, 89, 187, 188 and 191, important for Pak2 association (Agopian et al., 2006; O'Neill et al., 2006) are shaded. Bars mark the locations of other proposed Nef domains, including the myristoylation signal (Geyer et al., 2001), the PxxP SH3-binding region (Saksela et al., 1995), the putative CD4 binding site (Grzesiek et al., 1996), and the putative binding sites for thioesterase (Cohen et al., 2000), and the endocytic machinery components  $\beta$ -COP (Piguet et al., 1999a), adaptor proteins AP-1/2/3 (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998a) and vacuolar ATPase (Lu et al., 1998). The Clade B consensus is from the LANL sequence database (August 2004).

CD4 downregulation, was made in the background of NL4-3 Nef and was included as a negative control (Bresnahan et al., 1998; Craig et al., 1998; Greenberg et al., 1998a). We also included the EEEE<sub>65</sub>/AAAA (E4A) mutant of NL4-3 Nef which is defective for MHC-I downregulation but efficiently downregulates CD4 (Piguet et al., 2000). CD4 downregulation activity was highly conserved among primary Nefs, with all 10 clones showing at least some CD4 downregulation activity. Furthermore, 9 out of 10 primary clones downregulated CD4 at least as strongly as NL4-3 Nef. Six of these clones downregulated CD4 more strongly than NL4-3 Nef ( $p < 0.05$  by Student's two-tailed  $t$  test). Only one primary clone, MACS3 brain-derived Nef clone 16P, had weaker CD4 downregulation activity than NL4-3 Nef ( $p < 0.05$ ). Nef 16P also had less than

half the activity of the other primary Nef clones ( $p < 0.001$ ). MACS3 brain-derived Nef clone 16N strongly downregulated CD4, despite an apparent lower expression level suggested by Western blot (Fig. 3B). Moderately lower levels of Nef 16N may be sufficient for strong CD4 downregulation activity, since this function is induced even at lower concentrations of Nef (Fig. 3C and (Liu et al., 2001)). Alternatively, Nef 16N may be expressed at levels comparable to those of the other Nef alleles but poorly recognized by the polyclonal sera. Overall, there was no significant difference in CD4 downregulation activity between brain and lymphoid Nef clones. Together, these data demonstrate that CD4 downregulation is a highly conserved phenotype of primary Nef proteins from brain and lymphoid tissues of late stage AIDS patients.

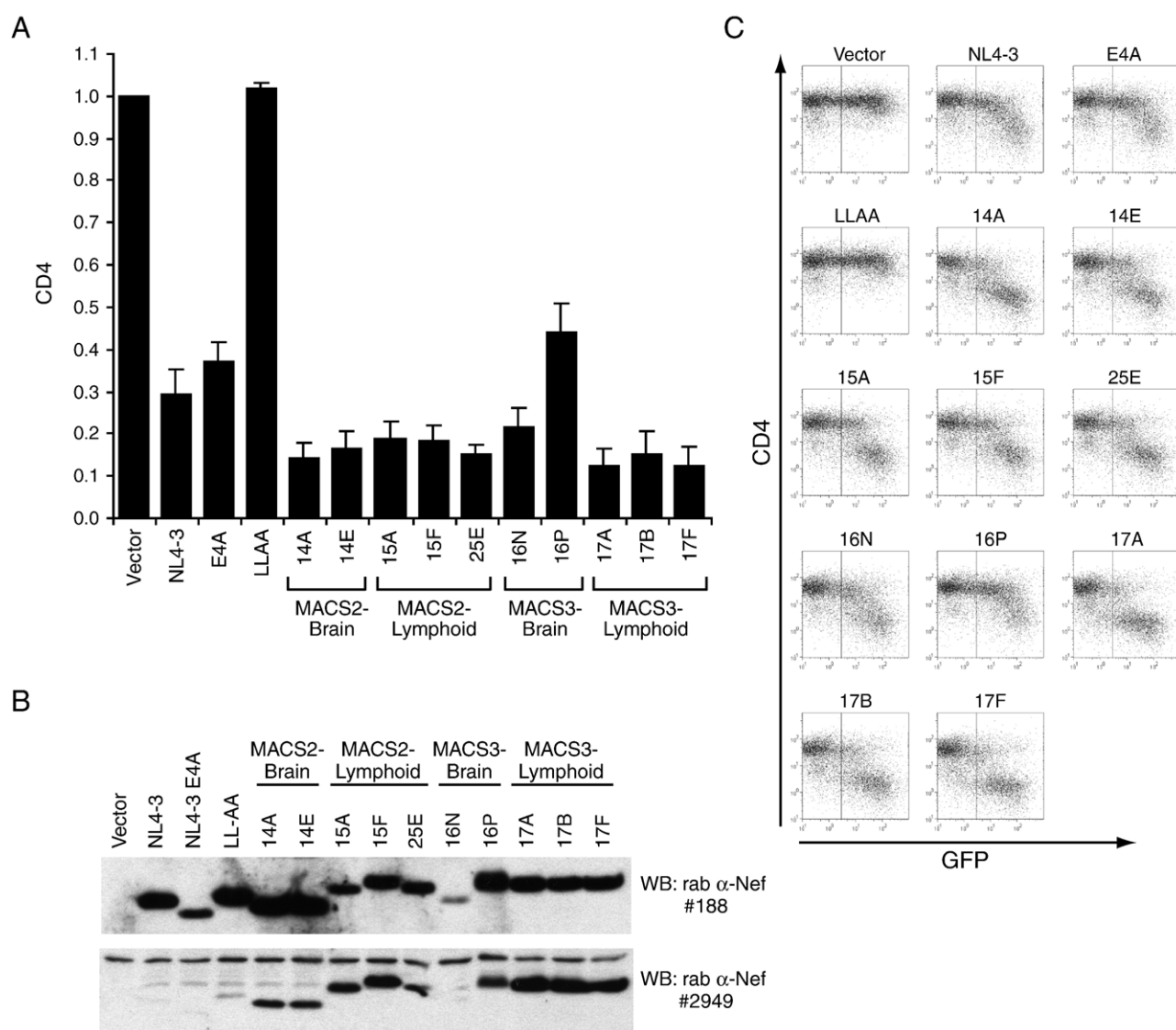


Fig. 3. Downregulation of cell surface CD4 is conserved in primary Nef alleles from brain and lymphoid tissues. (A) Quantitation of flow cytometric analysis of cell surface CD4 in HIJ cells co-transfected with pCDNA3-EGFP and either pCR3.1 (Vector) or pCR3.1-Nef plasmids as indicated. Relative surface CD4 was calculated from the geometric mean PE fluorescence in GFP-positive cells as described in Materials and methods and is shown relative to GFP-positive vector-transfected cells. Averages are from 3 independent experiments. Error bars represent standard deviations. (B) Nef expression in HIJ cells assayed for CD4 downregulation is confirmed by Western blot. Lysates were split and identical membranes were blotted in parallel with either rabbit anti-Nef #188 (raised to Bru) or rabbit anti-Nef #2949. (C) Primary FACS data for flow cytometric analysis of cell surface CD4 in transfected HIJ cells. Vertical lines indicate the threshold for GFP positive cells. Data shown are representative of three independent experiments.

*All tissue-derived Nefs downregulate MHC-I but brain-derived Nefs have reduced MHC-I downregulation*

To examine the ability of brain and lymphoid Nefs to downregulate MHC-I, we co-transfected Nef and GFP plasmids into Jurkat-T-Antigen cells and analyzed cells for surface expression of HLA-ABC by flow cytometry. MHC-I downregulation by Nef is generally less efficient than CD4 downregulation, with higher Nef expression levels required to induce MHC-I downregulation. Therefore, we only analyzed high-expressing cells, marked by high GFP levels. As a negative control, we included the EEEE<sub>65</sub>/AAAA (E4A) mutant of NL4-3, which has

been shown to abolish MHC-I downregulation (Piguet et al., 2000). All primary Nef clones downregulated MHC-I (Fig. 4). However, as a group, brain-derived Nef clones (14A, 14E, 16N, 16P) induced less MHC-I downregulation than lymphoid-derived Nef clones (15A, 15F, 25E, 17A, 17B, 17F) (Wilcoxon rank sum test, two-sided  $p$  value=0.01). Furthermore, all lymphoid-derived Nef clones downregulated MHC-I more strongly than did NL4-3 Nef ( $p$  values <0.05 by Student's  $t$  test for each Nef versus NL4-3). MACS2 brain Nef alleles were expressed at levels comparable to MACS2 lymphoid Nef alleles, suggesting that their reduced capacity to downregulate MHC-I is due to a specific functional defect (Fig. 4B). However, MACS3

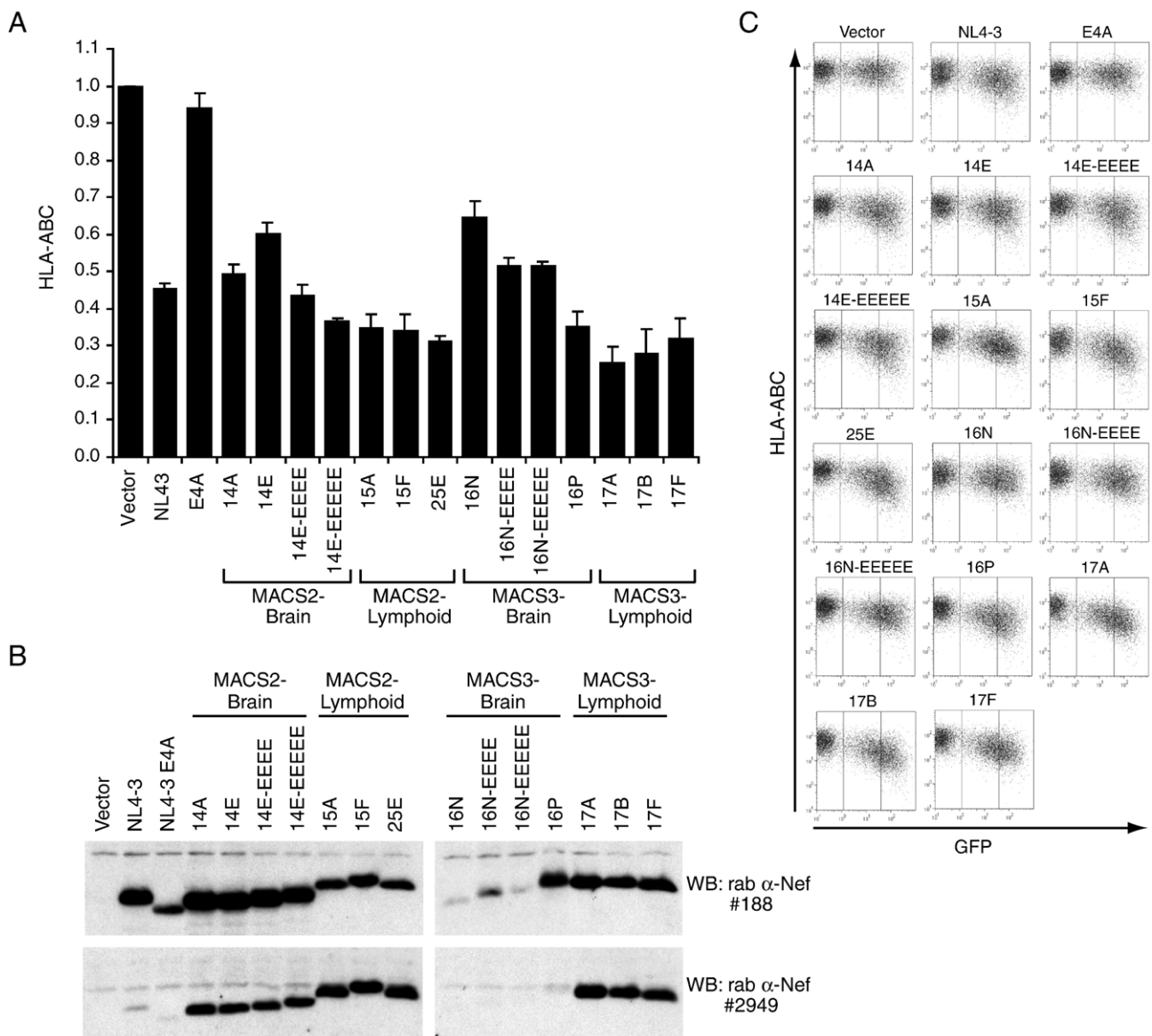


Fig. 4. Downregulation of cell surface MHC-I by primary Nef proteins from brain and lymphoid tissues. (A) Quantitation of flow cytometric analysis of cell surface HLA-ABC in Jurkat T-Antigen cells co-transfected with pCDNA3-EGFP and either pCR3.1 (Vector) or pCR3.1-Nef plasmids as indicated. Relative surface HLA-ABC was calculated from the geometric mean PE fluorescence in GFP-high-expressing cells as described in Materials and methods and is shown relative to GFP-high vector-transfected cells. Averages are from 2 to 5 independent experiments. Error bars represent standard deviations. (B) Nef expression in Jurkat T-Antigen cells used for MHC-I downregulation assays confirmed by Western blot. Lysates were split and identical membranes were blotted in parallel with either rabbit anti-Nef #188 (raised to Bru) or rabbit anti-Nef #2949. (C) Primary FACS data for flow cytometric analysis of cell surface MHC-I in transfected Jurkat T-Antigen cells. Vertical lines indicate the thresholds for GFP-negative, GFP-low and GFP-high expressing cells. Data shown are representative of two to five independent experiments.

brain Nef expression levels appeared to be lower than MACS3 lymphoid Nef expression levels (Fig. 4B and data not shown). Therefore, it is unclear whether the reduced ability of MACS3 brain Nef clones to downregulate MHC-I is due to a specific functional defect or simply to lower expression levels. Nevertheless, these data demonstrate that while MHC-I downregulation is conserved in primary brain- and lymphoid-derived Nef clones, brain-derived Nefs are reduced in their ability to downregulate MHC-I relative to lymphoid Nefs in these 2 late stage AIDS patients.

*Motifs KEEE- or EKEE- at the PACS-1 binding site represent brain-specific signature patterns and contribute to reduced MHC-I downregulation*

Subtle differences in the PACS-1 binding site, which is important for MHC-I downregulation (Blagoveshchenskaya et al., 2002; Piguet et al., 2000), were observed in brain- versus lymphoid-derived Nef clones (Fig. 2). Comparison of all

sequences across the data set from both patients using Viral Epidemiology Signature Pattern Analysis (VESPA) identified only one brain-specific pattern in Nef clones from both patients. A high frequency of Nef clones unique to brain had only 3 glutamic acids (EKEE<sub>-65</sub> or KEEE<sub>-65</sub>) in the PACS-1 binding site (Table 1). In contrast, the majority of Nef sequences from lymphoid tissue had 5 glutamic acids (EEEE<sub>-65</sub>). Representative of this difference, brain-derived Nef clones 14A, 14E and 16N have either the KEEE- or EKEE- motifs in their PACS-1 binding site, while 5 of 6 representative lymphoid Nef clones have 5 glutamic acids. The KEEE- or EKEE- sequences found in these brain-derived Nef clones are rare among Clade B Nef sequences, with only 0.88% or 0.44% of Nef sequences in the LANL database containing these sequences, respectively (Table 2). Therefore, we hypothesized that the KEEE- or EKEE- motifs represent a rare brain-specific signature pattern in these 2 patients that results in reduced ability to downregulate MHC-I.

To determine if the EKEE or KEEE motifs influence the ability of the primary Nef clones to downregulate MHC-I,

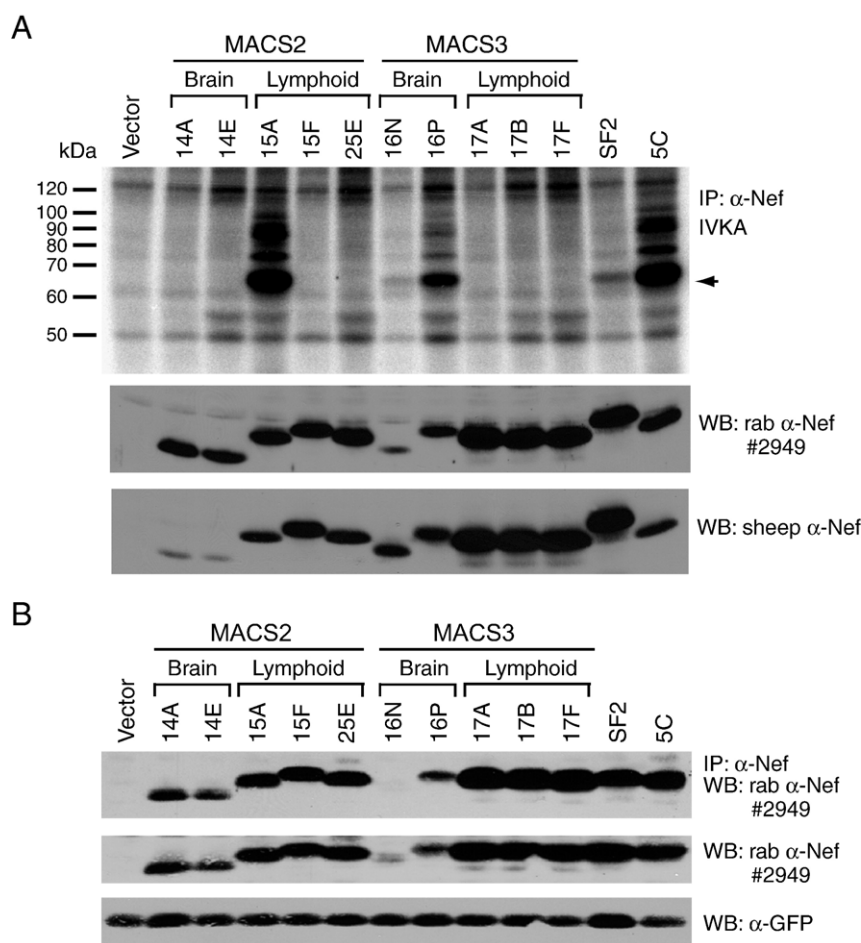


Fig. 5. Association of primary HIV-1 Nef proteins with activated Pak2 is highly variable. (A) In vitro kinase assay (IVKA). 293T cells transiently expressing GFP and the indicated Nef proteins were immunoprecipitated with sheep-anti-Nef (raised to SF2 Nef) and assayed by IVKA (top panel) as described in Materials and methods. SF2 Nef and MACS2 5C Nef, previously demonstrated to strongly activate Pak2 (Arora et al., 2000; Agopian et al., 2006), are included as positive controls. An arrowhead indicates the 62 kDa band corresponding to Pak2. Whole cell lysates used for IVKA immunoprecipitations were immunoblotted with rabbit-anti-Nef antibody #2949 or sheep anti-Nef (bottom panels). (B) 293T cells transiently expressing GFP and the indicated Nef proteins were immunoprecipitated with sheep-anti-Nef and Western blotted with rabbit anti-Nef #2949 to confirm efficient Nef immunoprecipitation (top panel). Input whole cell lysates were immunoblotted with anti-Nef antibody #2949 (middle panel) or anti-GFP (bottom panel).



Table 1  
Frequency of motifs at PACS-1 binding site in tissue-derived Nef sequences<sup>a</sup>

Patient	Brain	Lymphoid
MACS2	18/18 KEEE-(14A, 14E)	1/24 EEEEEK 23/24 EEEEE (15A, 15F, 25E)
MACS3	6/8 EKEE-(16N) 2/8 EEEE-(16P)	19/34 EQEEE (17F) 15/34 EEEEE (17A, 17B)
Total	18/26 KEEE- 6/26 EKEE- 2/26 EEEE-	38/58 EEEEE 19/58 EQEEE 1/58 EEEEEK

<sup>a</sup> Nef sequences unique to brain were compared to sequences from lymph node and spleen. VESPA analysis indicated signature sequences at positions 62–66 which distinguished brain-derived Nef sequences from lymphoid-derived Nef sequences. Sequence frequencies in each tissue are shown. Representative Nef clones used in functional assays are in parentheses.

mutations were made in the MACS2FL-14E and MACS3FL-16N backgrounds to restore the more common EEEE motif to these Nef proteins. Additionally, a fifth glutamic acid was inserted into the PACS-1 binding sites of these Nef clones to determine if an increased number of glutamic acids in the PACS-1 binding site enhances MHC-I downregulation activity. Nef mutant 14E-EEEE downregulated MHC-I more strongly than Nef 14E ( $p < 0.01$  by Student's  $t$  test) and Nef 14E-EEEEEE was even more potent relative to parental MACS2FL-14E ( $p < 0.001$ ) (Fig. 4A). In fact, there was no significant difference in downregulation of MHC-I by 14E-EEEEEE versus MACS2 lymphoid-derived Nef clones 15A and 15F, which contain the EEEEE motif in their PACS-1 binding sites. Nef 16N-EEEE also downregulated MHC-I more strongly than did the parental clone 16N. However, Western blot analyses suggest that this Nef is expressed at higher levels than clone 16N. Therefore, it is not possible to conclude whether the mutation at the PACS-1 binding site specifically increased MHC-I downregulation activity. However, Nef 16N-EEEEEE appeared to be expressed at levels similar to those of 16N, but downregulated MHC-I more strongly than 16N ( $p < 0.01$ ). Since the expression of 16N appears to be low, it is difficult to compare its MHC-I downregulation activity directly to that of the MACS3 lymphoid-derived Nef clones. Nevertheless, the 14E and 16N mutagenesis data support the hypothesis that 5 glutamic acids in the PACS-1 binding site result in the strongest downregulation of MHC-I, while the KEEE or EKEE motifs reduce MHC-I downregulation relative to the more common EEEE motif. Therefore, the reduced MHC-I downregulation activity of the brain-derived Nef clones in these 2 patients is likely a consequence of their KEEE or EKEE motifs and can be enhanced by mutation of these motifs to the more potent EEEE or EEEEE motifs.

*Pak2 association is variable, and does not correlate with brain or lymphoid origin of Nef sequences*

To determine whether association with activated Pak2 is conserved in primary brain and lymphoid-derived Nef proteins, representative Nef clones were tested for their ability to immunoprecipitate activated Pak2 by in vitro kinase assay (IVKA) (Fig. 5A). SF2 Nef and MACS2-LN isolate Nef clone

5C were used as positive controls (Agopian et al., 2006; Foster et al., 2001). 5C Nef associates with a very high amount of activated Pak2, while SF2 Nef is commonly used as a positive control for this assay (Arora et al., 2000; Foster et al., 2001; Luo and Garcia, 1996; Sawai et al., 1994). Of 10 tissue-derived Nef clones tested, 7 clones did not coimmunoprecipitate detectable Pak2 activity (Fig. 5A). Two tissue-derived Nef clones (MACS2LN-15A and MACS3FL-16P) coimmunoprecipitated more activated Pak2 than did the positive control SF2 Nef. Nef clone 16N coimmunoprecipitated a small amount of activated Pak2. Although Western blots with three different polyclonal antibodies suggest that Nef clone 16N has a lower expression level relative to the other Nef clones (see Figs. 3B, 4B, and 5A), lower expression alone cannot account entirely for the small amount of activated Pak2 immunoprecipitated by Nef 16N. To confirm that all Nef proteins were efficiently immunoprecipitated, anti-Nef Western blots were performed on anti-Nef immunoprecipitates (Fig. 5B). All Nef clones except 16N appeared to be efficiently immunoprecipitated. Since 16N appears to have a lower expression level than that of the other Nef alleles and is poorly recognized by the rabbit polyclonal antibody, it is likely that 16N was efficiently immunoprecipitated but its detection by Western blot was limited by a lower concentration of 16N in the cell lysate. Out of 10 tissue-derived Nef clones, we consider 2 to strongly associate with activated Pak2 and 1 to weakly associate with activated Pak2. Of MACS2 tissue-derived Nef clones, only 1 lymphoid-derived and no brain-derived Nef associated with activated Pak2. For MACS3, both brain-derived Nef clones but none of the lymphoid-derived clones associated with activated Pak2. These results demonstrate that the ability to coimmunoprecipitate activated Pak2 is not well conserved in primary Nef alleles from late stage AIDS patients. Furthermore, the ability of Nef sequences to associate with activated Pak2 did not correlate with either brain or lymphoid tissue origin in these 2 patients.

The ability of tissue-derived Nef clones to associate with activated Pak2 appeared to segregate with distinct branches of the phylogenetic trees (Fig. 6). MACS2 Nef alleles 15A and 5C immunoprecipitated a very high amount of activated Pak2 and share motif F<sub>89</sub>I<sub>187</sub>H<sub>188</sub>H<sub>191</sub> at residues critical for Pak2 association (Fig. 2 and (Agopian et al., 2006)), while clones 14A, 14E, 15F and 25E differ at some or all of these residues. Unlike most Clade B Nefs, all Nef clones from MACS2 have a phenylalanine at position 85, which is also critical for Pak2 association (Agopian et al., 2006). It is possible that in the

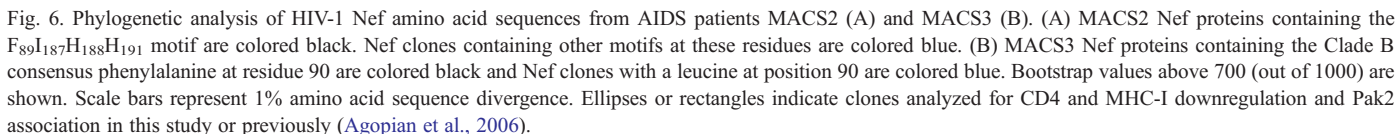
Table 2  
PACS-1 binding site motifs in clade B Nef sequences<sup>a</sup>

Nef 62–65	HIV Clade B ( $n=455$ )
EEEE-	62.7%
EDEE-	12.1%
KEEE-	0.88%
EKEE-	0.44%
EEEE	9.27%
Other <sup>b</sup>	14.61%

<sup>a</sup> Clade B Nef sequences deposited in the LANL database.

<sup>b</sup> Includes approximately 23 low-frequency variants.

Residue 90 of Nef is a phenylalanine in the clade B consensus sequence. F<sub>90</sub> interacts with the RT loop of SH3 domains (Lee et al., 1996; Lim et al., 1997) and mutation of this residue or its corresponding residue in SIV, F122, reduced or abolished association of Nef with Hck and NAK/Pak2 (Khan et al., 1998; Manninen et al., 1998). Therefore, the F<sub>90</sub>L variation in most MACS3 Nefs (shown in blue in Fig. 6B) would be expected to abolish Pak2 association. Barring other deleterious mutations, only the MACS3 alleles with F<sub>90</sub> (shown in black in Fig. 6B)



have the potential for Pak2 association. Thus, about one-third of all Nefs from patients MACS2 and MACS3 are predicted to associate with activated Pak2 based on sequence analysis.

## Discussion

This study provides evidence for distinct compartmentalization of *nef* genes from brain and lymphoid tissues of 2 patients with AIDS and HIV-1-associated dementia. CD4 downregulation activity was highly conserved among primary Nefs from brain and lymphoid tissues; 9 of 10 Nef clones downregulated CD4 strongly, in most cases more strongly than NL4-3 Nef, and one downregulated CD4 weakly. MHC-I downregulation was also conserved in these primary Nef clones. However, MHC-I downregulation by brain-derived Nefs was weaker than downregulation by lymphoid-derived Nefs. The motifs KEEE- or EKEE- at the PACS-1 binding site represented brain-specific signature patterns in these 2 patients that contributed to the reduced MHC-I downregulation ability of brain-derived Nefs that contained these motifs. In contrast to CD4 and MHC-I downregulation, Pak2 association was not well conserved. Three of 10 tissue-derived Nefs coimmunoprecipitated detectable activated Pak2, and only 2 of these were strongly associated with Pak2. Thus, CD4 and MHC-I downregulation are highly conserved Nef functions, while Pak2 association is variable in Nef clones from these 2 late stage AIDS patients.

*Nef* genes from viruses isolated from brain by coculture with PBMC were more closely related to sequences amplified from lymphoid tissue than from brain tissue, suggesting that viral selection or adaptation occurred during coculture. Thus, future studies of brain-specific genes should avoid isolating virus by co-culture with PBMC. Four brain-derived MACS3 sequences were identical to sequences from lymph node. These *nef* sequences could represent HIV-1 neuroinvasion during late stage AIDS after the blood–brain barrier has been disrupted, contamination by blood vessels that contain blood-derived or lymphoid Nef sequences, or archived ancestral sequences. Two main hypotheses have been proposed regarding the source of HIV-1 divergence within the CNS (McCrossan et al., 2006): (1) divergence is a result of early entry followed by isolation and genetic divergence; and (2) HIV-1 in the brain is a subpopulation of macrophage-tropic virus present in macrophages throughout the body, representing a macrophage-specific rather than brain-specific viral subpopulation. Thus, these 4 *nef* sequences amplified from brain that are similar to lymphoid-derived Nefs could represent archival HIV sequences in the brain present prior to genetic divergence or sequences from replicating macrophage-tropic subset of virus that is also present in lymphoid tissues.

CD4 downregulation activity was highly conserved in brain and lymphoid Nef alleles, consistent with previous reports that found 75–100% conservation of CD4 downregulation activity in Nefs from rapid progressors and late stage AIDS patients (Casartelli et al., 2003; Michael et al., 1995; Ratner et al., 1996; Tobiume et al., 2002). Brain-derived MACS3 Nef 16P was the only Nef with reduced CD4 downregulation. In Nef 16P, the WL<sub>58</sub> CD4 binding site and the motifs in the flexible loop critical

for association with the endocytic machinery are all conserved (Geyer et al., 2001). However, 16P contains the variation P<sub>122</sub>S. Mutation of proline122 dramatically affects both downregulation of CD4 and association with thioesterase (Cohen et al., 2000; Liu et al., 2000). Therefore, the P<sub>122</sub>S change may account for the reduced CD4 downregulation activity of Nef 16P.

By increasing the efficiency of viral particle release and subsequent engagement of CD4 receptors on target cells, downregulation of cell surface CD4 is one mechanism through which Nef enhances viral infectivity (Lama, 2003; Lama et al., 1999; Ross et al., 1999). CD4 downregulation is the most dramatic Nef function, and possibly the most important. Therefore, it is not surprising that this function is so highly conserved in our primary Nef alleles. It is important to note the strong conservation of CD4 downregulation in the brain, where target cells such as macrophages and microglia have very low CD4 levels. Our data, together with previous reports showing that even low levels of CD4, similar to those found in primary macrophages, significantly reduce the infectivity of HIV particles (Cortes et al., 2002) and that viruses expressing Nef alleles defective in CD4 downregulation exhibit delayed replication kinetics and reduced infectivity in primary macrophages (Brown et al., 2004) suggest that Nef-mediated CD4 downregulation is a critically important function in both high-CD4-expressing lymphocytes and low-CD4-expressing macrophages. These data underscore the importance of CD4 downregulation in the HIV life cycle.

All tissue-derived Nef alleles downregulated MHC-I. This finding is consistent with previous reports showing 90–100% conservation of this activity in progressing and late stage AIDS patients (Casartelli et al., 2003; Tobiume et al., 2002). The preservation of MHC-I downregulation in Nefs from these late stage AIDS patients suggests that MHC-I downregulation is an important function in the life cycle of HIV-1. However, in the 2 AIDS patients studied here, MHC-I downregulation by brain-derived Nefs was weaker than downregulation by lymphoid-derived Nefs. Furthermore, we demonstrated that motifs KEEE- or EKEE- at the PACS-1 binding site represented brain-specific signature patterns in these patients. Mutational analysis confirmed that KEEE<sub>-65</sub> or EKEE<sub>-65</sub> contributed to the reduced MHC-I downregulation ability of brain-derived Nefs from patients MACS2 and MACS3. To our knowledge, this is the first report of a functional difference between brain- and lymphoid-derived Nefs.

The motifs KEEE<sub>-65</sub> or EKEE<sub>-65</sub> are found in only 1.32% of 455 Clade B Nefs in the LANL database and only 1.2% of 1643 Clade B Nefs in the database of Foster and colleagues (O'Neill et al., 2006). Since these rare motifs were found at a high frequency in the brain but not lymphoid tissues of the 2 AIDS patients studied here, we hypothesized that these motifs might represent brain-specific signatures in some individuals with AIDS. In one previous report, 5 Nef clones derived from in vitro infection of PBMC with HIV-1 isolated from the cerebrospinal fluid of an AIDS dementia patient were found to have the motif KEEE (McPhee et al., 1998). In contrast, all 12 sequences amplified directly from uncultured brain tissue by Saksena et al. and the 2 sequences amplified directly from uncultured brain



tissue by Li et al. contain the common EEEE motif (Li et al., 1992; Saksena et al., 1997). Furthermore, 10 of 11 sequences amplified directly from brain by Van Marle et al. contain EEEE or EDEE motifs (van Marle et al., 2004). Therefore, EKEE and KEEE are associated with brain-derived Nefs in only a subset of AIDS patients. A search of the database of 1643 Nef alleles (O'Neill et al., 2006) revealed that 6 of 11 Nef sequences with motif KEEE were isolated from PBMC or plasma RNA (Asamitsu et al., 1999; Fang et al., 1999, 2001; Hahn et al., 2003; Kirchhoff et al., 1999), and 3 of 9 sequences with motif EKEE were isolated from PBMC (Asamitsu et al., 1999), while the source of the other 6 was not specified (Cho, and Ahn, direct submission, 2005 and Ashton, L et al unpublished, 2002). Therefore, the EKEE and KEEE motifs are not excluded from blood or lymphoid tissues nor are they a common brain signature pattern. Since immune surveillance is reduced in the CNS compared to non-CNS tissues, the brain may be a more permissive tissue site for maintenance of Nef alleles with weaker MHC-I downregulation.

In contrast to CD4 and MHC-I downregulation, Pak2 association was not well conserved in primary Nef alleles from 2 late stage AIDS patients. Only 3 of 10 tissue-derived Nefs immunoprecipitated Pak2. We found a higher frequency of Nef alleles positive for Pak2 activation (5 of 7) in Nef alleles from primary viral isolates UK1-Br, MACS1-Br, MACS1-Sp, MACS2-LN (5C), MACS3-br (6I), MACS3-LN (7D) and slow progressor  $\Delta$ 32-Blood [(Agopian et al., 2006; Gorry et al., 2001) and unpublished data]. Other studies also found higher rates of association with activated Pak2 among primary Nef alleles. In one study, 3 of 5 subtype B Nefs were positive for Pak2 activation and a second study found that 1 of 2 subtype B and 5 of 5 subtype E HIV-1 Nefs activated Pak2 (Foster et al., 2001; Huang et al., 1995; O'Neill et al., 2006). Furthermore, 5 of 5 Group O and N and 3 of 6 SIVcpz Nefs were strongly positive for Pak2 activation (Kirchhoff et al., 2004). The methods used by Kirchhoff et al appear to be more sensitive for detecting Pak2 association, since NL4-3 Nef coimmunoprecipitates a stronger band of Pak2 activity in their assay compared to ours (Arora et al., 2000; Luo and Garcia, 1996). Our IVKA detects Nef association with endogenous Pak2, whereas the IVKA used by Kirchhoff et al. detects association with exogenous Pak2 in cells cotransfected with activated Cdc42V12 (Pulkkinen et al., 2004).

The significance of the low frequency of Pak2 association in primary Nef alleles in our study remains unclear. Compilation of results of previous studies of Pak2 association suggests a frequency of 80% (16/20) positive in HIV-1 Nef alleles and 100% (7/7) positive in SIV Nef alleles (Foster et al., 2001; Kirchhoff et al., 2004; Luo and Garcia, 1996; O'Neill et al., 2006). An important distinction between our study and previous studies is the number of independent *nef* alleles amplified by limiting dilution from each patient and our selection, based on phylogenetics, of representative alleles to be tested in functional assays. Other studies that analyzed Nefs from several patients typically selected only one or a few alleles representing each patient. Further studies examining large numbers of Nef clones from several patients are needed to determine the true proportion

of Nef alleles that efficiently associate with and/or activate Pak2 in vivo.

If the true frequency of Nef alleles positive for Pak2 association in vivo is indeed low, then what is the significance of Nef–Pak2 association during the course of HIV infection? Evidence of compensation and co-variation between Nef residues critical for association with Pak2 suggests that Nef-mediated activation of Pak2 confers a selective advantage during the course of HIV infection (Agopian et al., 2006; O'Neill et al., 2006). Nef-mediated activation of Pak2 might be critical for infection of particular cell types during a specific stage(s) of infection. In the present study, tissue samples from patients MACS2 and MACS3 were collected during autopsy and therefore represent end-stage AIDS. If Pak2 activation is indeed critical for enhancement of T cell activation, as previously proposed (Fackler et al., 1999; Lu et al., 1996; Vilhardt et al., 2002; Wei et al., 2005; Wolf et al., 2001), then this function might be particularly important during transmission and/or early stages of infection when resting T cells are important targets for infection in genital mucosa and gut-associated lymphoid tissues (Pope and Haase, 2003). Nef residues important for Pak2 activation are also targets for CTLs (Los Alamos National Laboratories Database). Thus, the high level of variation at these sites in MACS2 could also reflect CTL escape. Further studies are needed to determine if the frequency of Nef alleles positive for Pak2 activation differs between specific types of infected cells or during the course of infection. The primary Nef alleles described here will facilitate future studies to investigate the importance of Nef–Pak2 association and other Nef functions for HIV-1 replication and pathogenesis.

## Materials and methods

### *Amplification of HIV-1 Nef alleles from autopsy tissue*

Genomic DNA was extracted from autopsy samples of uncultured brain, lymph node and spleen tissue from patients MACS2 and MACS3 using the Gentra Puregene DNA Purification kit (Gentra). Patients MACS2 and MACS3 were participants in the Chicago Component of the Multicenter AIDS Cohort Study who had late stage AIDS and HIV-1-associated dementia (Gorry et al., 2001). Quantification of HIV-1 *gag* copy number by real-time PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and primers CCR5-933R (5'-TGAAGATAAGCCTCACAGCCCT-3'), CCR5-778F (5'-AGGTCTTCATTACACCTGCAGCTC-3'), CCR5 probe (5'-CAGTCAGTATCAATTCTGGAAGAATTCCAGACAT-3'), 793-*gag* F (5'-GGTGCAGAGCGTCAGTATTAAG-3'), 911-*gag* R (5'-AGCTCCCTGCTTGCCCAT-3'), and 835-*gag* probe (5' TGGGAAAAAATTCGGTTAAGGCCAGGG-3'). Cycling conditions were 95 °C (15 min), followed by 45 cycles of 95 °C (15 s), 60 °C (1 min), 25 °C (2 min). Copies of HIV-1 *gag* per million cells were 3,460 and 2,713 in 2 different sections of MACS2 frontal lobe, 21,683 in MACS2 lymph node, 6,129 in MACS2 spleen, 1,645 in MACS3 frontal lobe and 252,104 in MACS3 lymph node. The HIV-1 *nef* gene was amplified by nested PCR performed on 1  $\mu$ l



of undiluted genomic DNA (bulk PCR) or at limiting dilution. Dilutions were determined to be limiting when an average of 3 of 10 PCR reactions were positive. For brain genomic DNA, limiting dilutions ranged from 1:2 to 1:8. For lymph node and spleen DNA, dilutions ranged from 1:50 to 1:200. Maximally conserved primer binding sites were selected. To reduce effects of selective amplification, 2 different sets of nested PCR primers were used. Positions of the primer sequences in the HIV-1 HXB2 genome are given in parentheses. For the first set, outer primers were **Nef1** (5'-GCCCCAAGGAATAGAAGAAG-3') (8411–8430) and **Nef2** (5'-GCACTCAAGGCAAGCTTTATTGAGGC-3') (9631–9605). The Nef2 primer was previously published (Huang et al., 1995). Inner primers included the restriction sites *Bam*HI and *Cla*I (underlined) and were **Nef3b.5** (5'-TTAGTGAACGGATCCTTAGCACTTATCTGGG-3') (8466–8496) and **Nef4.5** (5'-GCGGAAAGTCCCTTGTCACATCGATGTCA-3') (9446–9415). For the second set, outer primers were **Nef5'b** (5'-GCAGTAGCTGAGGGGACAGATAGGGTTATAG) (8685–8705) and **Nef2**. Inner primers were **HI5'** (5'-CGTCTAGAACATACCTAGAAGAATAAGACAGG) (8748–8768) and **NFkB3'** (5'-AAAGTCCCCAGCGGAAAGTCCC) (9456–9435). All reactions were performed with Pfu polymerase (Stratagene). The HI5' primer was previously published (Huang et al., 1995). The cycling conditions for the 50 µl first-round reaction were 94 °C (2 min), followed by 30 cycles of 94° (15 s), 57 °C (15 s), 68 °C (3 min), and then the final elongation step at 72 °C (7 min). 1 µl of the first-round PCR product was used as template for the second-round reaction. The cycling conditions for the second-round primer set were the same except that hybridization was performed at 55 °C. PCR products were purified using the Qiaquick PCR purification kit and were either sequenced directly (represented by numbers, e.g. LN-15) or cloned into the pCR3.1 expression vector and sequenced (represented by numbers and letters, e.g. LN-15A). From each tissue, 11 to 34 independent sequences were obtained.

The nucleotide and amino acid sequences from MACS2 frontal lobe (DQ885391–DQ885408), MACS2 lymph node (DQ358015–DQ358036), MACS2 spleen (DQ358037–DQ358047), MACS3 frontal lobe (DQ885409–DQ885420), and MACS3 lymph node (DQ885421–DQ885454) have been submitted to the GenBank sequence database.

#### *Amplification of HIV-1 Nef alleles from viral isolates*

The primary HIV-1 viruses MACS2-LN, MACS3-br and MACS3-LN were isolated from patients MACS2 and MACS3 and characterized as described (Gorry et al., 2001). Briefly, autopsy lymph node and brain tissue samples were homogenized and cocultured with CD8-depleted PBMC. Supernatants testing positive for reverse transcriptase activity were filtered and stored at –80 °C. From cell pellets of cultured PBMC infected with viral supernatants, DNA was extracted using a Qiagen DNeasy kit. Nef alleles were amplified and cloned from 1 µl undiluted genomic DNA as described above.

The nucleotide and amino acid Nef sequences of viral isolate-derived clones MACS2LN-5C (DQ357219), MACS2LN-5F

(DQ358012), MACS2LN-5G (DQ358013), MACS2LN-5H (DQ358014), MACS3br-6I (DQ357220), and MACS3LN-7D (DQ357221) have been submitted to GenBank.

#### *Phylogenetic analysis*

Sequences from each independent limiting dilution PCR reaction and each clone from bulk PCR were included in the phylogenetic analyses. Alignments were made using Clustal W. Bootstrapped phylogenetic trees were created by the neighbor-joining method using Clustal X.

#### *Plasmids and mutagenesis*

The NL4-3 *nef* allele was amplified from the pNL4-3 proviral plasmid by PCR with primers Nef3b.5 and Nef 4.5 and inserted into pCR3.1 by TA cloning. The SF2 *nef* allele was cloned into the *Eco*RI site of vector pCDNA3.1 (Foster et al., 2001). The pCR3.1 vector lacking an insert was used for mock transfections. Mutations were created using the QuikChange site-directed mutagenesis kit (Stratagene) and all mutants were confirmed by sequencing.

#### *Cell culture*

293T cells and HIJ cells (HeLa cells retrovirally transduced to express high levels of CD4), provided by David Kabat (Kabat et al., 1994), were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 100 µg/ml penicillin/streptomycin. Jurkat-T-Antigen (J-TAg) cells (Northrop et al., 1993), provided by Heinrich Gottlinger, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 µg/ml penicillin/streptomycin.

#### *CD4 and MHC-I downregulation assays*

For CD4 downregulation assays, HIJ cells were co-transfected with *nef* and pCDNA3-EGFP expression plasmids using Lipofectamine 2000 (Invitrogen). Cells were harvested after 28 h and stained for 30 min at 4 °C with saturating amounts of PE-conjugated mouse anti-CD4 antibody (BD Pharmingen). Stained cells were analyzed by flow cytometry and dead cells were excluded by forward and side scatter (Becton Dickinson FACScan). The geometric mean of CD4-PE fluorescence intensity was calculated for transfected (GFPpos) and untransfected (GFPneg) cell populations. To calculate the relative mean fluorescence intensity (relMFI) of each transfected sample, the following formula was used:  $\text{relMFI}_{\text{sample}} = ((\text{MFI}_{\text{GFPpos}}) / (\text{MFI}_{\text{GFPneg}})_{\text{sample}}) / ((\text{MFI}_{\text{GFPpos}}) / (\text{MFI}_{\text{GFPneg}})_{\text{Vector}})$ . This formula corrects for slight staining variation between stained samples and expresses the CD4 surface levels relative to the mock-transfected negative control.

MHC-I downregulation assays were performed by co-transfecting Jurkat T-Antigen cells with *nef* and pCDNA3-EGFP plasmids using Lipofectamine 2000. Cells were activated 4 h later with 1 µg/ml PHA-L and 50 ng/ml PMA. Cells were harvested after 28 h and stained with a saturating amount of PE-

conjugated mouse anti-HLA-ABC antibody (BD Pharmingen). Stained cells were analyzed by flow cytometry as described above. The relative MFI of HLA-ABC-PE fluorescence was calculated for high-GFP-expressing (GFP<sup>high</sup>) using the formula described above.

### Western blots

Aliquots of cells used for CD4 and MHC-I downregulation assays were lysed in Cold Lysis Buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, Complete protease inhibitors (Roche)). Lysates were centrifuged at 14,000 rpm for 20 min at 4 °C to remove cell debris. Equal amounts of total protein were run on 12% SDS-PAGE gels and analyzed by Western blotting with rabbit anti-Nef #2949 (AIDS Research and Reference Reagent Program), rabbit anti-Nef Bru #188 (Zazopoulos and Haseltine, 1992), or sheep anti-Nef SF2 (Foster et al., 2001). Aliquots of cell lysates used for the IVKAs were tested for Nef expression by Western blot with sheep polyclonal anti-Nef SF2 serum (1:2000 dilution) as described (Foster et al., 2001) or rabbit anti-Nef #2949 (1:2000 dilution).

### In vitro kinase assays (IVKAs)

IVKAs were performed as previously described (Foster et al., 2001). Briefly, 293T cells transfected (Lipofectamine 2000, Invitrogen) with Nef and pN1GFP plasmids were lysed in IVKA Lysis Buffer (50 mM Tris pH 8.0, 100 mM NaCl, 10% glycerol, 0.5% IGEPAL CA-630) (Sigma), 1 mM EDTA, 25 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM beta-glycerophosphate, 1 mM PMSF, 25 mM benzamidin and Complete protease inhibitors (Roche). Cleared whole cell lysates (600 µg) were immunoprecipitated with sheep-anti-Nef serum. Kinase reactions were performed on immunoprecipitates in the presence of [ $\gamma$ <sup>32</sup>P] ATP and separated by 10% SDS-PAGE gels. Dried gels were exposed to a phosphorimager screen (Packard).

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